METHODS OF PREVENTING OR TREATING CELL-MIGRATION MEDIATED CONDITIONS OR DISEASES

Introduction

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Background of the Invention

The ability of cells to form cell contacts, adhere to the extracellular matrix, change morphology, and migrate is essential for development, wound healing, metastasis, cell survival and the immune response. These events depend on the binding of integrin to the extracellular matrix, and assembly of focal adhesions, which are complexes of scaffolding and signaling proteins organized by adhesion to the extracellular matrix (Critchley (2000) *Curr. Opin. Cell Biol.* 12:133-139; Burridge and Chrzanowska-Wodnicka (1996) *Annu. Rev. Cell Dev. Biol.* 12:463-518; Schwartz and Ginsberg (2002) *Nature Cell Biol.* 4:E65-E68). Phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) regulates interactions between these proteins, including the interaction of vinculin with actin and talin (McNamee, *et al.* (1993) *J. Cell Biol.* 121:673-678; Berditchevski, *et al.* (1997) *J. Biol. Chem.* 272:2595-2598; Chong, *et al.* (1994) *Cell* 79:507-513; Gilmore and Burridge (1996) *Nature* 381:531-535; Steimle, *et al.* (1999) *J. Biol. Chem.* 274:18414-18420; Martel, *et al.* (2001) *J. Biol. Chem.* 276:21217-21227). The binding of talin to β-integrin is strengthened by $PtdIns(4,5)P_2$, suggesting that the basis of focal adhesion assembly is regulated by this lipid mediator (Martel, *et al.* (2001) *supra;* Janmey (1994) *Annu. Rev.*

Physiol. 56:169-191). Further, it has been suggested that PIPKIγ661/talin association is important for targeting PIPKIγ661 to focal adhesions (Di Paolo, *et al.* (2002) *Nature* 420:85-89; Ling, *et al.* (2002) *Nature* 420:89-93).

Summary of the Invention

One aspect of the present invention is a method for identifying an agent that modulates the activity of Type I phosphatidylinositol phosphate kinase isoform γ661 (PIPKIγ661). The method involves contacting PIPKIγ661 with a test agent in the presence of at least one selected protein and detecting the activity of PIPKIγ661. A change in the activity of PIPKIγ661 as compared to a control is indicative of said agent modulating the activity of PIPKIγ661. In one preferred embodiment, the agent modulates the activity of Src thereby modulating the activity of PIPKIγ661. In another preferred embodiment, the agent modulates the activity of FAK thereby modulating the activity of PIPKIγ661.

Another aspect of the present invention is a method for identifying an agent that modulates cell focal adhesion assembly. The method involves contacting a cell which lacks active PIPKIγ661 or which overexpresses PIPKIγ661 with a test agent and measuring the adherence of said cell to a surface. A difference in the adherence of the cell to the surface in the presence of the test agent as compared to the adherence of the cell to the surface in the absence of the test agent is indicative of the agent modulating cell focal adhesion assembly.

A further aspect of the present invention is a method of preventing or treating a cell migration-mediated condition or disease in a subject. The method involves

administering to a subject an effective amount of an agent that modulates the activity of PIPKIγ661 or cell focal adhesion assembly in a cell which lacks active PIPKIγ661 or which overexpresses PIPKIγ661 so that at least one sign or symptom of a cell migration-mediated condition or disease is prevented or treated.

Brief Description of the Drawings

Figure 1 illustrates the epitope-tagged constructs and relative focal adhesion (FA) targeting. c-Myc-tagged constructs are chimeras of PIPKIα and PIPKIγ tail.

Figure 2 demonstrates the inhibition of talin binding to integrin with PIPKIγ661 peptides PN (diamonds), pY644 (squares), pY649 (circles) and pY644/649 (triangles).

Figure 3 illustrates constructs of PIPKIγ661 truncations or tyrosine mutations.

Detailed Description of the Invention

Adherence to extracellular matrix stimulates the generation of PtdIns(4,5)P_2 (McNamee, *et al.* (1993) *supra;* Chong, *et al.* (1994) *supra*) and may modulate focal adhesion assembly around clusters of integrins (Martel, *et al.* (2001) *supra;* Janmey (1994) *supra*). Type I phosphatidylinositol phosphate kinase isoforms (PIPKIs) generate the lipid messenger PtdIns(4,5)P_2 (Anderson, *et al.* (1999) *J. Biol. Chem.* 274:9907-9910; Kunz, *et al.* (2000) *Mol. Cell* 5:1-11). The PIPKIγ transcripts are alternatively spliced messenger RNA, resulting in the PIPKIγ635 and PIPKIγ661 isoforms that differ by a 26-amino-acid carboxy-terminal extension (Ishihara, *et al.* (1998) *J. Biol. Chem.* 273:8741-8748).

It has now been found that the PIPKIγ661 isoform is specifically targeted to focal adhesions by an association with talin. PIPKIγ661 was tyrosine phosphorylated by focal adhesion-associated kinase signaling, increasing both the activity of phosphatidylinositol phosphate kinase and its association with talin.

Immunolocalization studies indicated that PIPKIγ661 was targeted to focal adhesions. To determine whether the C-terminal 26 amino acids of PIPKIγ661 were sufficient for targeting, these residues were fused to green fluorescent protein (GFPγ26). The GFPγ26 fusion protein was poorly targeted to focal adhesions, indicating that other regions within PIPKIγ661 were also required. To define these constraints, chimeras of PIPKIα, an enzyme not targeted to focal adhesions, were produced. The PIPKIα/γ chimeras contained 178 residues from the C-terminus of PIPKIγ661 (PIPKIα/γ661), the 178 residues from the C-terminus of PIPKIγ661 lacking the 26 C-terminal amino acid sequences (PIPKIγ635) or 26 amino acids from the C-terminus of PIPKIγ661 fused to the C-terminus of PIPKIα (PIPKIα/γ26) (Figure 1). PIPKIα/γ661 and PIPKIα/γ26 were targeted to focal adhesions, whereas PIPKIα/γ635 was not. Enhanced targeting of PIPKIα chimera constructs containing the C-terminal 26 residues reflects structural or functional features of the phosphatidylinositol phosphate kinases, such as dimer formation, which are required for activity at membranes (Kunz, *et al.* (2000) *supra;* Rao, *et al.* (1998) *Cell* 94:829-839).

Two approaches were used to identify proteins that specifically interact with PIPKIγ661. Haemagglutinin (HA)-

tagged PIPKIγ661 or PIPKIγ635 were expressed in HEK 293T cells, and isolated by immunoprecipitation. As numerous focal adhesion proteins are tyrosine phosphorylated, the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibodies. A tyrosine-phosphorylated protein of relative molecular mass 230,000 (M_r 230K) was selectively immunoprecipitated with PIPKIγ661. This protein was identified as talin (Pasquale, *et al.* (1986) *Proc. Natl Acad. Sci. USA* 83:5507-5511; DeClue and Martin (1987) *Mol. Cell Biol.* 7:371-378). Vinculin, focal adhesion-associated kinase (FAK) and tensin did not interact with PIPKIγ661. Only the PIPKIα/γ chimeras that target to focal adhesions associated with talin. To confirm the talin-PIPKIγ661 interaction, talin was immunoprecipitated from HEK293T cells overexpressing PIPKIγ661 or PIPKIγ635. Talin co-precipitated with PIPKIγ661, but not PIPKIγ635. It is known that endogenous PIPKIγ splice isoforms are widely expressed in various cell lines. When endogenous PIPKIγ was immunoprecipitated from a variety of cells types, talin association was consistently observed.

A yeast two-hybrid screen using the 178-residue C-terminal region of PIPKIγ661 as bait resulted in the isolation of 12 talin clones. This demonstrated a direct interaction, and narrowed the interacting region to residues 150 to 480 in the talin head region. This region encompasses a portion of F1 and the F2 and F3 regions of talin's band 4.1, ezrin, radixin, and moesin (FERM) homology domain. The talin FERM domain also contains regions shown to interact with other focal adhesion proteins (Calderwood, *et al.* (1999) *J. Biol. Chem.* 274:28071-28074; Rees, *et al.* (1990) *Nature* 347:685-689;

Chisti, *et al.* (1998) *Trends Biochem. Sci.* 23:281-282; Calderwood, *et al.* (2002) *J. Biol. Chem.* 277:21749-21758).

These data demonstrate that the PIPKIγ661-talin association is important for targeting PIPKIγ661 to focal adhesions. This interaction was further substantiated by the immunofluorescent colocalization of PIPKIγ661 and talin in NRK cells. As PtdIns(4,5)P_2 modulates the association of talin with β-integrin and other focal adhesion proteins (Gilmore and Burridge (1996) *supra;* Martel, *et al.* (2001) *supra;* Janmey (1994) *supra*), it was determined whether the PIPKIγ661-talin interaction regulates the focal adhesion assembly of talin. Moderate overexpression of PIPKIγ661 resulted in substantially larger talin-containing focal adhesions in 43% of cells compared to non-transfected cells. This indicated that PIPKIγ661 modulates talin assembly into focal adhesions. High overexpression of PIPKIγ661, but not PIPKIγ635, resulted in a loss of talin from focal adhesions in 49% of the transfected cells. These data indicate that increasing levels of PtdIns(4,5)P_2 at focal adhesions cause talin assembly and disassembly. Overexpression of PIPKI isoforms reorganizes actin from stress fibers to cortical actin filaments (Anderson, *et al.* (1999) *supra;* Ishihara, *et al.* (1998) *supra*). Thus, phenotypes observed by high overexpression of PIPKIγ661 may also affect focal adhesions indirectly.

Expression of a kinase-inactive, also referred to herein as kinase-dead, PIPKIγ661 mutant (PIPKIγ661kd) resulted in a loss of talin targeting to focal adhesions in 70% of the cells expressing high levels. This further indicated a role for PtdIns(4,5)P_2 in talin assembly into focal adhesions.

During adhesion and spreading, cells assemble focal adhesions rapidly, providing a good model to define the role of PIPKIγ661 in membrane and focal adhesion assembly of talin. PIPKIγ661 facilitated the targeting of talin to the plasma membrane in adhering cells. This was independent of PIP kinase activity, as PIPKIγ661kd also facilitated targeting. Significantly, PIPKIγ661 facilitated the assembly of talin-containing focal adhesions in spreading cells, and this was dependent on PIP kinase activity. These data show that PIPKIγ661 regulates both plasma membrane targeting and the efficient assembly of talin into focal adhesions.

The association between PIPKIγ661 and talin was also regulated by adhesion to type I collagen. In co-immunoprecipitation of talin with PIPKIγ661 from HEK293T cells in suspension versus cells adherent to type I collagen, it was found that talin strongly associated with PIPKIγ661 in adherent cells, but not in suspended cells. However, in suspended cells, the PIPKIγ661 association with talin was restored when cells were stimulated with lysophosphatidic acid (LPA) or platelet-derived growth factor (PDGF).

Tyrosine phosphorylation of focal adhesion proteins is stimulated by adhesion, and is important for focal adhesion assembly (Cary and Guan (1999) *Front. Biosci.* 4:D102-D113). PIPKIγ661 was tyrosine phosphorylated, and this was dependent upon PIP kinase activity. Tyrosine phosphorylation was also stimulated upon cell adhesion to type I collagen. FAK is an important tyrosine kinase at focal adhesions and is activated by LPA (Rodriquez-Fernandez and Rozengurt (1998) *J. Biol. Chem.* 273:19321-19328). To determine FAK's role in tyrosine phosphorylation

of PIPKIγ661, dominant active FAK (CD2FAK)(Chan, *et al.* (1994) *J. Biol. Chem.* 269:20567-20574) was co-expressed with each of the PIPKIγ isoforms. This induced a 4-fold increase in tyrosine phosphorylation of PIPKIγ661, but not PIPKIγ635, indicating that focal adhesion targeting was required for tyrosine phosphorylation of PIPKIγ661. Tyrosine phosphorylation of PIPKIγ661kd was also stimulated by co-expression of CD2FAK, although to a lesser extent. Co-expression of the dominant-negative FAK related non-kinase (FRNK), which blocks endogenous FAK activity, diminished tyrosine phosphorylation of PIPKIγ661. FRNK also inhibited adhesion-stimulated PIPKIγ661 tyrosine phosphorylation. The FAK-induced tyrosine phosphorylation of PIPKIγ661 correlated with a substantial increase in its association with talin. Tyrosine phosphorylation of PIPKIγ661 may facilitate association with talin via the phosphotyrosine-binding site in the talin head (Calderwood, *et al.* (2002) *supra*). Significantly, CD2FAK, but not FRNK, specifically stimulated the activity of PIPKIγ661. These data indicate that FAK signaling stimulates both talin association and PIP kinase activity, resulting in localized generation of PtdIns(4,5)P_2 at focal adhesions.

A functional link between PIPKIγ661 and FAK was provided by the observation that PIPKIγ661kd, but not PIPKIγ661, disrupted targeting of FAK to focal adhesions. PIPKIγ661 and PIPKIγ661kd were both targeted to focal adhesions and co-localized with FAK. High expression of PIPKIγ661 did not affect FAK targeting. In contrast, the expression of PIPKIγ661kd resulted in complete loss of FAK targeting to focal adhesions in 39% of the expressing cells. A number of other focal adhesion proteins were

analyzed to determine the effect of PIPKIγ661 and PIPKI661γkd expression. From these studies, paxillin and vinculin were more resistant to changes induced by ectopic expression of PIPKIγ661 or PIPKI661γkd.

Further, PIPKIγ661 tyrosine phosphorylation stimulation by FAK was dependent upon both FAK phosphorylation on Tyr^{397} and FAK activity, since neither co-expressed Tyr^{397}->Phe or kinase dead mutants of FAK induced phosphorylation of PIPKIγ661. To narrow the phosphorylation sites of PIPKIγ661, the PIPKIα/γ chimeras were co-expressed with wild-type FAK in HEK293T cells, and tyrosine phosphorylation of these proteins was analyzed. Both PIPKIα/γ661 and PIPKIα/γ26 were phosphorylated by FAK, but not PIPKIα/γ635, indicating that the major FAK signaling phosphorylation sites were in the last 26 amino acids of PIPKIγ661. In parallel, tail-truncation mutants of PIPKIγ661 were constructed (Figure 3) and the ability of the mutants to be phosphorylated by co-expressed FAK was examined. PIPKIγ-W, which lacked both Tyr^{644} and Tyr^{649}, was not phosphorylated while PIPKIγ-Y containing Tyr^{644} was phosphorylated, but to a lesser extent. PIPKIγ-E retained both Tyr^{644} and Tyr^{649} and was phosphorylated similar to the wild-type kinase. This indicated that Tyr^{644} and Tyr^{649} were potential FAK signaling phosphorylation sites. Further, Tyr to Phe point mutants at positions 644 and 649 of PIPKIγ661 were constructed and tyrosine phosphorylation was assayed in cells. PIPKIγTyr^{649}->Phe was still phosphorylated by FAK at the levels similar to wild-type PIPKIγ661. However, PIPKIγTyr^{644}->Phe showed a dramatic reduction in phosphorylation, indicating that Tyr^{644} was the FAK signaling phosphorylation site. These data, combined with the weak phosphorylation of PIPKIγ-Y

indicated that the residues from Ser^{645} to Glu^{658} were required for efficient phosphorylation of PIPKIγ661.

In the FAK signaling pathway, Src family tyrosine kinases bind to and are activated by FAK, playing a fundamental role in focal adhesion organization (Parsons, *et al.* (2000) *Oncogene* 19:5606-5613; Cobb, *et al.* (1994) *Mol. Cell. Biol.* 14:147-155; Schaller, *et al.* (1994) *Mol. Cell. Biol.* 14:1680-1688; Schaller, *et al.* (1999) *Mol. Cell. Biol.* 10:3489-3505). To determine if Src family kinases phosphorylate PIPKIγ661, Src-specific inhibitor PP2 was employed. It was found that PP2, but not the inactive analogue PP3, dramatically inhibited both basal and FAK-induced tyrosine phosphorylation of PIPKIγ661, indicating that Src family kinases phosphorylated PIPKIγ661. Furthermore, the results from *in vitro* kinase assays demonstrated that c-Src directly phosphorylated recombinant PIPKIγ661 tail, while FAK does not. Consistent with these results, both recombinant PIPKIγTyr^{644}->Phe and PIPKIγ$Tyr^{644/649}$->Phe were not efficiently phosphorylated by c-Src *in vitro* compared with wild-type. PIPKIγTyr^{649}->Phe is efficiently phosphorylated by c-Src. These results demonstrate that Tyr^{644} of PIPKIγ661 is the direct phosphorylation site of c-Src. Two-dimensional-phosphopeptide mapping indicated that both Tyr^{644} and Tyr^{649} were phosphorylated by c-Src indicating that the phosphorylation of Tyr^{649} may be depend upon the phosphorylation of Tyr^{644}.

The mechanism of PIPKIγ661 phosphorylation by Src was analyzed to determine whether there was an interaction between Src and PIPKIγ661. It was found that c-Src co-immunoprecipitated with PIPKIγ661. It has been shown that

Src binding to FAK activates Src and both phosphorylate substrates synergistically (Schaller (2001) *Biochim. Biophys. Acta* 1540:1-21). The role of FAK in mediating Src phosphorylation of PIPKIγ661 was further examined. Co-expression of FAK and c-Src with PIPKIγ661 enhanced tyrosine phosphorylation and Src association with PIPKIγ661. Co-expression of FAK-Tyr^{397}->Phe lacking the docking site for Src interaction inhibited the association of PIPKIγ661 and c-Src. PIPKIγ635 and the PIPKIγ661 Tyr to Phe mutants could bind c-Src, indicating that c-Src interacts with PIPKIγ at a site distinct from the last 26 amino acids of PIPKIγ661 and independent of Tyr^{644} or Tyr^{649} phosphorylation. Since FAK does not interact with PIPKIγ661, these results indicate that FAK recruits c-Src to PIPKIγ661 by binding to the FERM domain of talin. This is further supported by the poor phosphorylation and weak talin interaction of PIPKIγ-Y although it contains Tyr^{644}.

Tyrosine phosphorylation of focal adhesion proteins regulates focal adhesion assembly by creating protein-protein interaction sites (Ridley and Hall (1994) *EMBO J.* 13:2600-2610; Chrzanowska-Wodnicka and Burridge (1994) *J. Cell Sci.* 107:3643-3654; Barry and Critchley (1994) *J. Cell Sci.* 107:2033-2045). As shown herein, increased PIPKIγ661 tyrosine phosphorylation by FAK signaling was correlated with PIPKIγ661/talin interaction. Talins interaction with PIPKIγ661 phosphorylation-defective mutants was analyzed to define whether PIPKIγ661 tyrosine phosphorylation was necessary for the interaction with talin. Compared with wild-type PIPKIγ661, PIPKIγ-W did not interact with talin, PIPKIγ-Y maintained weak talin association, and PIPKIγ-E had identical talin association. The tyrosine point mutants,

PIPKIγTyr^{644}->Phe and PIPKIγTyr$^{644/649}$->Phe both showed substantially diminished talin association, while PIPKIγTyr^{649}->Phe retained talin association.

Targeting requirements were further examined by analyzing the focal adhesion localization of PIPKIγ661 mutants in NRK cells. PIPKIγ-W lost focal adhesion targeting, PIPKIγ-E targeted to focal adhesions and co-localized with talin similar to wild-type PIPKIγ661. PIPKIγ-Y partially retained focal adhesion targeting. Mutants lacking phosphorylation had reduced talin association and lost focal adhesion targeting, while PIPKIγTyr^{649}->Phe localized to focal adhesions similar to wild-type. Quantification revealed a correlation between focal adhesion targeting, talin association, and phosphorylation of PIPKIγ661.

Talin plays a key role in integrin-mediated signaling processes by linking integrins to the actin cytoskeleton and regulating integrin activation (Liu, *et al.* (2000) *J. Cell Sci.* 113:3563-3571; Calderwood, *et al.* (2002) *J. Biol. Chem.* 277:21749-21758). The F3 lobe of the talin FERM domain, structurally homologous to a phosphotyrosine binding-like (PTB-like) domain, binds to the β-integrin cytoplasmic tail (Calderwood, *et al.* (2002) *supra;* Garcia-Alvarez, *et al.* (2003) *Mol. Cell.* 11:49-58). As demonstrated herein, this region also binds the last 26 amino acids of PIPKIγ661. Accordingly, it was determined whether PIPKIγ661 tyrosine phosphorylation directly enhanced its interaction with talin. In addition, it was examined whether PIPKIγ661 would displace β-integrin from talin in a phosphorylation dependent manner.

Talin interactions were assessed by GST pull-down using recombinant GST-talin head, His-PIPKIγ635 C-terminus, His-PIPKIγ661 wild-type or Tyr to Phe mutant C-terminus, and His-β1-integrin tail. His-PIPKIγ661 Tyr to Phe mutants showed identical binding to talin compared to wild-type, indicating that the decreased *in vivo* talin association directly resulted from the lack of phosphorylation. Furthermore, it was found that His-PIPKIγ661, but not His-PIPKIγ635, competed with His-β1-integrin for binding to GST-talin in a dose-dependent manner. Peptides containing the PIPKIγ661/talin binding sequence (Di Paolo, *et al.* (2002) *supra*) were designed to include phosphorylated Tyr^{644}, phosphorylated Tyr^{649}, and dual phosphorylated Tyr^{644} and Tyr^{649}. The phosphorylated peptides displaced PIPKIγ661 from talin more efficiently than the non-phosphorylated peptide in HEK293T cell lysates. *In vitro*, the non-phosphorylated peptide poorly displaced His-β1-integrin from GST-talin, while the phosphorylated peptides competed with a 20-fold higher affinity.

From structural studies of integrin binding to talin, a conserved tryptophan in β-integrin tail is positioned near two basic residues, Lys^{357} and Arg^{358} of talin F3 lobe (Garcia-Alvarez, *et al.* (2003) *supra*). It was demonstrated that Trp^{642} of PIPKIγ661 is a key residue required for talin binding (Di Paolo, *et al.* (2002) *supra*), positioning the Tyr^{644} adjacent to Lys^{357} and Arg^{358} of talin. To determine if the binding affinity of the phosphorylated peptides was due to these two basic residues, each was mutated to glutamine. Both Lys^{357}->Gln and Arg^{358}->Gln mutants abolished His-β1-integrin binding, but unexpectedly, had no effect on His-PIPKIγ661 binding. These data demonstrate that PIPKIγ661

binds to talin on an overlapping yet distinct site compared to β1-integrin. The role of PIPKIγ661 phosphorylation was examined by using phosphorylated peptides to compete His-PIPKIγ661 binding to wild-type or mutant GST-talin. It was found that Lys^{357}->Gln retained high affinity binding for the Tyr^{644}-phosphorylated peptide but lower affinity for the Tyr^{649}-phosphorylated peptide. Arg^{358}->Gln showed a loss of affinity for both Tyr^{644}- and Tyr^{649}-phosphorylated peptides. This indicated that both phosphorylated residues require Arg^{358}->Gln for high affinity binding but the Tyr^{649} appears to also require Lys^{357}->Gln. The enhanced association of the phosphorylated peptides with talin indicated that the β1-integrin tail may bind to talin with increased affinity when phosphorylated at the ^{780}Trp-Asp-Thr^{783} and ^{785}Asn-Pro-Ile-Tyr^{788} (SEQ ID NO:1) motif, since the Trp-Asp-Thr aligns with Tyr^{644} of PIPKIγ661. Accordingly, Arg^{358}->Gln would coordinate the phosphorylated Thr similar to Tyr^{644} of PIPKIγ661. To further analyze this, β1-integrin peptides phosphorylated at Thr of Trp-Asp-Thr and at Tyr of Asn-Pro-Ile-Tyr (SEQ ID NO:1) were synthesized and used to compete integrin tail binding from talin. The phosphorylated Trp-Asp-Thr peptide did not increase binding affinity to talin. However, the phosphorylated Asn-Pro-Ile-Tyr (SEQ ID NO:1) peptide showed lower binding affinity, indicating that phosphorylation of the Tyr residue poorly interacted with GST-talin, consistent with previous reports (Garcia-Alvarez, *et al.* (2003) *supra;* Barsukov, *et al.* (2003) *J. Biol. Chem.* M303850200).

The interaction between talin and Src-phosphorylated PIPKIγ661 was further analyzed by computer modeling the talin F3 lobe (Garcia-Alverez, *et al.* (2003) *supra*) with the dual phosphorylated PIPKIγ661 peptide. In this model,

Tyr^{644} of PIPKIγ661 is positioned to interact with Lys^{357} and Arg^{358} of talin. Unlike the integrin sequence, Pro^{646} of PIPKIγ661 created a turn that positioned Tyr^{649} directly adjacent to Lys^{357} of talin. This is consistent with results provided herein that showed that the Lys^{357}->Gln mutant lost the enhanced binding to Tyr^{649}-phosphorylated peptide. As Lys^{357} and Arg^{358} of talin are key residues for binding phosphorylated Tyr^{644} and Tyr^{649} of PIPKIγ661, it was determined if Arg or Lys residues are conserved in other FERM domains. The Lys or Arg residues were found at the same position in other FERM domains including Moesin, Radixin, Ezrin, and Band 4.1 (Garcia-Alverez, *et al.* (2003) *supra*), indicating structurally conserved binding between FERM domains and phosphorylated tyrosine residues.

The interaction between PIPKIγ661 and talin appeared to be critical for focal adhesion assembly. This is supported by observations that a β-integrin binding site regulated by PtdIns(4,5)P_2 is located in the head region of talin within the FERM domain (Martel, *et al.* (2001) *supra*; Calderwood, *et al.* (1999) *supra*; Rees, *et al.* (1990) *supra*; Chisti, *et al.* (1998) *supra*). Proteins containing the FERM domain, such as protein 4.1 and talin, interact with integral membrane proteins, such as glycophorin or integrins, and these interactions are modulated by PtdIns(4,5)P_2 (Martel, *et al.* (2001) *supra*; Chisti, *et al.* (1998) *supra*; Anderson and Marchesi (1985) *Nature* 318:295-298; Hirao, *et al.* (1996) *J. Cell Biol.* 135:37-51). Therefore, the PIPKIγ661-talin interaction may be important in the initiation of focal adhesion assembly via regulation of integrin binding and other focal adhesion proteins by PtdIns(4,5)P_2 (Critchley (2000) *supra*; Burridge and Chrzanowska-Wodnicka (1996) *supra*; Schwartz and Ginsberg (2002) *supra*; Gilmore

and Burridge (1996) *supra*; Steimle, *et al.* (1999) *supra*; Martel, *et al.* (2001) *supra*).

While not wishing to be bound by any one mechanism of action, it is believed that upon clustering of integrins, talin and PIPKIγ661 are recruited to focal adhesions, inducing synthesis of PtdIns(4,5)P_2. Spatial generation of PtdIns(4,5)P_2 facilitates the recruitment and regulation of proteins such as vinculin, α-actinin and FAK. The formation of a complex consisting of PIPKIγ661, talin, FAK, and Src, may facilitate Src interaction with PIPKIγ661 and phosphorylate PIPKIγ661 at Tyr^{644}. Tyrosine-phosphorylation of PIPKIγ661 increases binding affinity to talin and displaces β-integrin. Thus, regulated and localized generation of PtdIns(4,5)P_2 facilitates the assembly and/or disassembly of focal adhesions. PIPKI activity is also regulated by small G proteins (McNamee, *et al.* (1993) *supra*; Chong, *et al.* (1994) *supra*; Honda, *et al.* (1999) *Cell* 99:521-532) and by phosphatidic acid, a product of phospholipase D (PLD)(Jenkins, *et al.* (1994) *J. Biol. Chem.* 269:11547-11554). PLD activity is required for actin stress fiber formation and α-actinin assembly at focal adhesions (Kam and Exton (2001) *Mol. Cell Biol.* 21:4055-4066). In many cells, adhesion is essential for survival and growth, which require phosphoinositol 3-kinase (PI3K) activity (Cary and Guan (1999) *supra*). PI3K is also modulated by FAK, and may be dependent upon PIPKIγ661 for its substrate. Consequently, generation of PtdIns(4,5)P_2 by PIPKIγ661 may also control the generation of messengers derived from PtdIns(4,5)P_2. Thus, PIPKIγ661 may be at a signaling branch point that modulates both focal adhesion assembly and signals emanating from focal adhesions. As PIPKIγ661 appears

to be central to focal adhesion assembly, agents which modulate the activity of PIPKIγ661 or events leading up to the phosphorylation of PIPKIγ661 (*i.e.*, FAK and Src interactions) would be useful as therapeutics for cell-migration mediated conditions or diseases.

Accordingly, one aspect of the present invention is a method for identifying an agent that modulates the activity of PIPKIγ661. The method involves contacting PIPKIγ661 with a test agent in the presence of at least one selected protein and detecting the activity of PIPKIγ661, wherein a change in the activity of PIPKIγ661 compared to a control is indicative of the agent modulating the activity of PIPKIγ661. In one embodiment of the present invention, the activity of PIPKIγ661 is defined by the binding interaction with talin. In this embodiment, the selected protein is talin. In another embodiment the activity of PIPKIγ661 is defined by the phosphorylation of PIPKIγ661 in the presence of the selected protein Src. Further, the phosphorylation of PIPKIγ661 may be determined in the presence of Src in combination with FAK. As used herein, an agent which modulates the activity of PIPKIγ661 includes an agent which stimulates, enhances or activates its activity as well as an agent which inhibits, reduces or decreases the activity of PIPKIγ661. It is contemplated that an agent, which modulates the activity of PIPKIγ661 may interact with either PIPKIγ661 or talin to modulate the interaction between PIPKIγ661 and talin or interact with PIPKIγ661, Src or FAK to modulate the phosphorylation of PIPKIγ661. An agent with interacts with Src or FAK modulates the activity of Src or FAK thereby modulating the activity of PIPKIγ661.

A PIPKIγ661 protein which may be used within the scope of the invention includes a full-length PIPKIγ661 (SEQ ID NO:2) or any fragment, homolog, or ortholog which binds to talin. In a preferred embodiment of the invention, a fragment of PIPKIγ661 encompasses the C-terminal 178 amino acid fragment of PIPKIγ661 (SEQ ID NO:3), the 25 C-terminal amino acid residues of PIPKIγ661 (Thr-Asp-Glu-Arg-Ser-Trp-Val-Tyr-Ser-Pro-Leu-His-Tyr-Ser-Ala-Arg-Pro-Ala-Ser-Asp-Gly-Glu-Ser-Asp-Thr; SEQ ID NO:4) or a fragment thereof (*e.g.*, Asp-Glu-Arg-Ser-Trp-Val-Tyr-Ser-Pro-Leu-His-Tyr-Ser-Ala-Arg; SEQ ID NO:5).

When detecting the binding of PIPKIγ661 to talin, a talin protein which may be used within the scope of the invention includes a full-length talin (SEQ ID NO:6) or any fragment which binds to PIPKIγ661. In a preferred embodiment of the invention, a fragment of talin encompasses the 450 N-terminal amino acid residues of talin (SEQ ID NO:7), residues 150-450 of talin (SEQ ID NO:8) which bind PIPKIγ661 or residues 206-435 of talin (SEQ ID NO:9) which bind β-integrin.

When detecting the phosphorylation of PIPKIγ661 by Src, a Src protein which may be used includes a full-length Src (SEQ ID NO:10) or any fragment which phosphorylates PIPKIγ661. Further, when the assay is carried out in the presence of FAK, a FAK protein may include a full-length FAK (SEQ ID NO:11) or any fragment which modulates the phosphorylation of PIPKIγ661 via Src.

A PIPKIγ661, Src, FAK or talin protein or fragment thereof may be derived from the native polypeptide sequence, as well as recombinantly-produced or chemically-synthesized polypeptides which function in a manner similar

to the reference molecule to achieve a desired result. Thus, a functional fragment of PIPKIγ661, Src, FAK or talin encompasses derivatives, homologues, orthologs and analogues of those polypeptides including any single or multiple amino acid additions, substitutions, and/or deletions occurring internally or at the amino or carboxy termini thereof so long as binding activity remains.

Methods for producing recombinant PIPKIγ661, Src, FAK or talin proteins are well-known in the art. In general, nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin are incorporated into a recombinant expression vector in a form suitable for expression of the proteins in a host cell. A suitable form for expression provides that the recombinant expression vector includes one or more regulatory sequences operatively-linked to the nucleic acids encoding PIPKIγ661, Src, FAK or talin in a manner which allows for transcription of the nucleic acids into mRNA and translation of the mRNA into the protein. Regulatory sequences may include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are known to those skilled in the art and are described in Goeddel D.D., ed., Gene Expression Technology, Academic Press, San Diego, CA (1991). It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transfected and/or the level of expression required.

A PIPKIγ661, Src, FAK or talin protein may be expressed not only directly, but also as a fusion protein with a heterologous polypeptide, *i.e.* a signal sequence for secretion and/or other polypeptide which will aid in the purification of PIPKIγ661, Src, FAK or talin. Preferably,

the heterologous polypeptide has a specific cleavage site to remove the heterologous polypeptide from PIPKIγ661, Src, FAK or talin.

In general, a signal sequence may be a component of the vector and should be one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For production in a prokaryote, a prokaryotic signal sequence from, for example, alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders may be used. For yeast secretion, one may use, *e.g.*, the yeast invertase, alpha factor, acid phosphatase leaders, the *Candida albicans* glucoamylase leader (EP 362,179), or the like (see, for example WO 90/13646). In mammalian cell expression, signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal may be used.

Other useful heterologous polypeptides which may be fused to PIPKIγ661, Src, FAK or talin include those which increase expression or solubility of the fusion protein or aid in the purification of the fusion protein by acting as a ligand in affinity purification. Typical fusion expression vectors include those exemplified herein (fusion vectors of c-Myc and HA) as well as pGEX vectors (Amersham Biosciences, Piscataway, NJ), pMAL and pTYB vectors (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia Biotech, Piscataway, NJ) which fuse glutathione-S-transferase, maltose E binding protein, intein/chitin binding domain or protein A, respectively, to the target recombinant protein.

PIPKIγ661, Src, FAK or talin is expressed in a cell by introducing nucleic acid sequences encoding PIPKIγ661, Src,

FAK or talin into a host cell, wherein the nucleic acids are in a form suitable for expression of PIPKIγ661, Src, FAK or talin in the host cell. Alternatively, nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin which are operatively-linked to regulatory sequences (*e.g.*, promoter sequences) but without additional vector sequences may be introduced into a host cell. As used herein, a host cell is intended to include any prokaryotic or eukaryotic cell or cell line so long as the cell or cell line is not incompatible with the protein to be expressed, the selection system chosen or the fermentation system employed.

Eukaryotic cell or cell lines which may be used to produce PIPKIγ661, Src, FAK or talin include mammalian cell lines as well as non-mammalian cells. Exemplary mammalian cell lines include, but are not limited to, those exemplified herein as well as CHO dhfr- cells (Urlaub and Chasin (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220), 293 cells (Graham, *et al.* (1977) *J. Gen. Virol.* 36:59) or myeloma cells like SP2 or NSO (Galfre and Milstein (1981) *Meth. Enzymol.* 73(B):3-46). A variety of non-mammalian eukaryotic cells may be used as well, including insect (*e.g,. Spodoptera frugiperda*), yeast (*e.g., S. cerevisiae, Schizosaccharomyces pombe, Pichia pastoris, Kluveromyces lactis, Hansenula polymorpha*, and *Candida albicans*), fungal cells (*e.g., Neurospora crassa, Aspergillus nidulins, Aspergillus fumigatus*) and plant cells.

While any prokaryotic cell may be used to produce PIPKIγ661, Src, FAK or talin, *Escherichia coli* is the most common prokaryotic expression system. Strains which may be used to maintain expression plasmids include, but are not limited to, JM103, JM105, and JM107. Exemplary *E. coli*

strains for protein production include W3110 (ATCC 27325), *E. coli B*, *E. coli* X1776 (ATCC 31537), *E. coli* BL21 (Amersham Biosciences, Piscataway, NJ), *E. coli* ER5266 (New England Biolabs, Beverly, MA) and *E. coli* 294 (ATCC 31446).

For production of PIPKIγ661, Src, FAK or talin in recombinant prokaryotic expression vectors it is contemplated that protein expression may be regulated by promoters such as the beta-lactamase (penicillinase) and lactose promoter systems (Chang, *et al.* (1978) *Nature* 275:615; Goeddel, *et al.* (1979) *Nature* 281:544), a tryptophan (trp) promoter system (Goeddel, *et al.* (1980) *Nucl. Acids Res.* 8:4057; EP 36,776) the tac promoter (De Boer, *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:21) or pL of bacteriophage 1. These promoters and Shine-Dalgarno sequence may be used for efficient expression of PIPKIγ661, Src, FAK or talin in prokaryotes.

Eukaryotic microbes such as yeast may be transformed with suitable vectors containing nucleic acids encoding PIPKIγ661, Src, FAK or talin. *Saccharomyces cerevisiae* is the most commonly studied lower eukaryotic host microorganism, although a number of other species already mentioned are commonly available. Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin, sequences for polyadenylation and transcription termination, and nucleic acid sequences encoding a selectable marker. Exemplary plasmids include YRp7 (Stinchcomb, *et al.* (1979) *Nature* 282:39; Kingsman, *et al.* (1979) *Gene* 7:141; Tschemper, *et al.* (1980) *Gene* 10:157), pYepSec1 (Baldari, *et al.* (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30:933-943),

pJRY88 (Schultz, *et al.* (1987) *Gene* 54:113-123), and pYES2 (INVITROGEN™ Corporation, San Diego, CA). These plasmids contain genes such as *trp1*, which provides a selectable marker for a mutant strain of yeast lacking the ability to grow in the presence of tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones (1977) *Genetics* 85:12). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable sequences for promoting PIPKIγ661, Src, FAK or talin expression in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman, *et al.* (1980) *J. Biol. Chem.* 255:2073) or other glycolytic enzymes (Hess, *et al.* (1968) *J. Adv. Enzyme Reg.* 7:149; Holland, *et al.* (1978) *Biochemistry* 17:4900), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further disclosed in EP 73,657.

When the host cell is from an insect (*e.g.*, *Spodoptera frugiperda* cells), expression vectors such as the baculovirus expression vector (*e.g.*, vectors derived from *Autographa californica* MNPV, *Trichoplusia ni* MNPV, *Rachiplusia ou* MNPV, or *Galleria ou* MNPV, as described in U.S. Patent Nos. 4,745,051 and 4,879,236) may be employed to express PIPKIγ661, Src, FAK or talin. In general, a baculovirus expression vector comprises a baculovirus genome containing nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin inserted into the polyhedrin gene at a

position ranging from the polyhedrin transcriptional start signal to the ATG start site and under the transcriptional control of a baculovirus polyhedrin promoter.

In plant cells, expression systems are often derived from recombinant Ti and Ri plasmid vector systems. In the cointegrate class of shuttle vectors, the gene of interest is inserted by genetic recombination into a non-oncogenic Ti plasmid that contains both the *cis*-acting and *trans*-acting elements required for plant transformation. Exemplary vectors include the pMLJ1 shuttle vector (DeBlock, *et al.* (1984) *EMBO J.* 3:1681-1689) and the non-oncogenic Ti plasmid pGV2850 (Zambryski, *et al.* (1983) *EMBO J.* 2:2143-2150). In the binary system, the gene of interest is inserted into a shuttle vector containing the *cis*-acting elements required for plant transformation. The other necessary functions are provided *in trans* by the non-oncogenic Ti plasmid. Exemplary vectors include the pBIN19 shuttle vector (Bevan (1984) *Nucl. Acids Res.* 12:8711-8721) and the non-oncogenic Ti plasmid pAL4404 (Hoekema, *et al.* (1983) *Nature* 303:179-180) and derivatives thereof.

Promoters used in plant expression systems are typically derived from the genome of plant cells (*e.g.*, heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (*e.g.*, the 35S RNA promoter of CaMV; the coat protein promoter of TMV).

In mammalian cells the recombinant expression vector may be a plasmid. Alternatively, a recombinant expression vector may be a virus, or a portion thereof, which allows for expression of a nucleic acid introduced into the viral nucleic acid. For example, replication-defective retroviruses, adenoviruses and adeno-associated viruses may be used. Protocols for producing recombinant retroviruses

and for infecting cells *in vitro* or *in vivo* with such viruses may be found in Current Protocols in Molecular Biology, Ausubel, F. M. *et al.* (eds.) John Wiley & Sons, (1996), Section 9 and other standard laboratory manuals. Examples of suitable retroviruses include, but are not limited to, pLJ, pZIP, pWE and pEM which are well-known to those skilled in the art. Examples of suitable packaging virus lines include, but are not limited to, ψCrip, ψCre, ψ2 and ψAm. The genome of adenovirus may be manipulated such that it encodes and expresses PIPKIγ661 or talin but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle (Berkner, *et al.* (1988) *BioTechniques* 6:616; Rosenfeld, *et al.* (1991) *Science* 252:431-434; Rosenfeld, *et al.* (1992) *Cell* 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 etc.) are well-known to those skilled in the art. Alternatively, an adeno-associated virus vector such as that taught by Tratschin, *et al.* ((1985) *Mol. Cell. Biol.* 5:3251-3260) may be used to express PIPKIγ661, Src, FAK or talin.

In mammalian expression systems, the regulatory sequences are often provided by the viral genome. Commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For example, the human cytomegalovirus IE promoter (Boshart, *et al.* (1985) *Cell* 41:521-530), HSV-Tk promoter (McKnight, *et al.* (1984) *Cell* 37:253-262) and β-actin promoter (Ng, *et al.* (1985) *Mol. Cell. Biol.* 5:2720-2732) may be useful in the expression of PIPKIγ661, Src, FAK or talin in mammalian cells. Alternatively, the regulatory sequences of the recombinant expression vector may direct expression of PIPKIγ661, Src,

FAK or talin preferentially in a particular cell-type, *i.e.*, tissue-specific regulatory elements may be used. Examples of tissue-specific promoters which may be used include, but are not limited to, the albumin promoter (liver-specific; Pinkert, *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji, *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci USA* 86:5473-5477), pancreas-specific promoters (Edlund, *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316; EP 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Camper and Tilghman (1989) *Genes Dev.* 3:537-546).

Nucleic acid sequences or expression vectors harboring nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin may be introduced into a host cell by standard techniques for transforming cells. Transformation or transfection are intended to encompass all conventional techniques for introducing nucleic acid into host cells, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, polyethylene glycol-mediated transformation, viral infection, *Agrobacterium*-mediated transformation, cell fusion, and ballistic bombardment. Suitable methods for transforming host cells may be found in Sambrook, *et al.* (Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold

Spring Harbor Laboratory Press (2000)) and other laboratory manuals.

The number of host cells transformed with a nucleic acid sequence encoding PIPKIγ661, Src, FAK or talin will depend, at least in part, upon the type of recombinant expression vector used and the type of transformation technique used. Nucleic acids may be introduced into a host cell transiently, or more typically, for long-term expression of PIPKIγ661, Src, FAK or talin, the nucleic acid sequence is stably integrated into the genome of the host cell or remains as a stable episome in the host cell. Plasmid vectors introduced into mammalian cells are typically integrated into host cell DNA at only a low frequency. In order to identify these integrants, a gene that contains a selectable marker (*e.g.*, drug resistance) is generally introduced into the host cells along with the nucleic acids of interest. Preferred selectable markers include those which confer resistance to certain drugs, such as G418 and hygromycin. Selectable markers may be introduced on a separate plasmid from the nucleic acids of interest or introduced on the same plasmid. Host cells transfected with nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin (*e.g.*, a recombinant expression vector) and a gene for a selectable marker may be identified by selecting for cells using the selectable marker. For example, if the selectable marker encodes a gene conferring neomycin resistance, host cells which have taken up the nucleic acid sequences of interest may be selected with G418 resistance. Cells that have incorporated the selectable marker gene will survive, while the other cells die.

A host cell transformed with nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin may be used for expressing PIPKIγ661, Src, FAK or talin for protein production or may be used in cell-based screening assays to identify agents which modulate cell adhesion. Further, a host cell transformed with nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin may be transformed with one or more nucleic acid sequences which serve as targets for PIPKIγ661, Src, FAK or talin.

Nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin may be introduced into cells growing in culture *in vitro* by conventional transformation techniques (*e.g.*, calcium phosphate precipitation, DEAE-dextran transfection, electroporation, etc.). Nucleic acids may also be transferred into cells *in vivo*, for example by application of a delivery mechanism suitable for introduction of nucleic acid into cells *in vivo*, such as retroviral vectors (see *e.g.*, Ferry, *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Kay, *et al.* (1992) *Hum. Gene Ther.* 3:641-647), adenoviral vectors (see *e.g.*, Rosenfeld (1992) *Cell* 68:143-155; Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816), receptor-mediated DNA uptake (see *e.g.*, Wu and Wu (1988) *J. Biol. Chem.* 263:14621; Wilson, *et al.* (1992) *J. Biol. Chem.* 267:963-967; U.S. Patent No. 5,166,320), direct injection of DNA uptake (see *e.g.*, Acsadi, *et al.* (1991) *Nature* 334:815-818; Wolff, *et al.* (1990) *Science* 247:1465-1468) or particle bombardment (see *e.g.*, Cheng, *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:4455-4459; Zelenin, *et al.* (1993) *FEBS Let.* 315:29-32).

Nucleic acid sequences encoding PIPKIγ661, Src, FAK or talin may be transferred into a fertilized oocyte of a non-human animal to create a transgenic animal which expresses

PIPKIγ661, Src, FAK or talin in one or more cell-types. A transgenic animal is an animal having cells that contain a transgene, wherein the transgene was introduced into the animal or an ancestor of the animal at a prenatal, *e.g.*, an embryonic, stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell-types or tissues of the transgenic animal. Exemplary examples of non-human animals include, but are not limited to, mice, goats, sheep, pigs, cows or other domestic farm animals. Such transgenic animals are useful, for example, for large-scale production of PIPKIγ661, Src, FAK or talin (gene pharming) or for basic research investigations.

A transgenic animal may be created, for example, by introducing a nucleic acid sequence encoding PIPKIγ661, Src, FAK or talin, typically linked to appropriate regulatory sequences, such as a constitutive or tissue-specific enhancer, into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Intron sequences and polyadenylation signals may also be included in the transgene to increase the efficiency of expression of the transgene. Methods for generating transgenic animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. A transgenic founder animal may be used to breed additional animals carrying the transgene. Transgenic animals carrying a transgene encoding PIPKIγ661, Src, FAK or talin may further be bred to other transgenic animals carrying other transgenes, *e.g.*, a

transgenic animal overexpressing PIPKIγ661 may be bred with a transgenic animal overexpressing talin.

Once produced, the PIPKIγ661, Src, FAK or talin may be recovered from culture medium or milk as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal. When PIPKIγ661, Src, FAK or talin is expressed in a recombinant cell other than one of human origin, the PIPKIγ661, Src, FAK or talin is free of proteins or polypeptides of human origin. However, it may be necessary to purify PIPKIγ661, Src, FAK or talin from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to PIPKIγ661, Src, FAK or talin. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The PIPKIγ661, Src, FAK or talin may then be purified from the soluble protein fraction. PIPKIγ661, Src, FAK or talin thereafter is purified from contaminant soluble proteins and polypeptides, as exemplified herein or with, for example, the following suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; chitin column chromatography, reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, SEPHADEX G-75; ligand affinity chromatography, and protein A SEPHAROSE columns to remove contaminants such as IgG.

In addition to recombinant production, PIPKIγ661, Src, FAK or talin or fragments thereof may be produced by direct peptide synthesis using solid-phase techniques (Merrifield

J. (1963) *J. Am. Chem. Soc.* 85:2149-2154). Protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Boston, MA). Various fragments of PIPKIγ661, Src, FAK or talin may be chemically-synthesized separately and combined using chemical methods to produce the full-length molecule.

Whether recombinantly-produced or chemically-synthesized, PIPKIγ661, Src, FAK or talin polypeptides or fragments thereof may be further modified for use in the screening methods of the invention. For example, the peptides or polypeptides may be glycosylated, phosphorylated or fluorescently-tagged using well-known methods. For example, Src may be phosphorylated prior to screening assays with PIPKIγ661.

Screening assays for identifying an agent which modulates the activity of PIPKIγ661 may be performed in any format that allows rapid preparation and processing of multiple reactions such as in, for example, multi-well plates of the 96-well variety. Stock solutions of the agents as well as assay components are prepared manually and all subsequent pipeting, diluting, mixing, washing, incubating, sample readout and data collecting is done using commercially available robotic pipeting equipment, automated work stations, and analytical instruments for detecting the signal generated by the assay.

In addition to PIPKIγ661 and Src/FAK or talin, a variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, *e.g.*, albumin, detergents, etc. which may be used to facilitate optimal protein-protein binding and/or reduce

non-specific or background interactions. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, and the like may be used. The mixture of components may be added in any order that provides for the requisite binding.

In a screening assay to identify an agent which modulates the activity of PIPKIγ661 as defined by the binding interaction between PIPKIγ661 and talin, the step of detecting the activity of PIPKIγ661 is carried out by detecting and measuring the binding of PIPKI-γ661 to talin. The detection and measurement of this binding interaction will be dependent on the type of screening assay performed and the labels used. Such screening assays to detect binding between two proteins in the presence of a test agent are well-known in the art. In a preferred embodiment of the present invention, a fluorescently labeled PIPKIγ661 peptide is used in a binding assay with talin or a fragment thereof to identify agents which modulate the PIPKIγ661 and talin interaction. An exemplary binding assay of this type was conducted using PIPKIγ661 peptides of the sequence Cys-Asp-Glu-Arg-Ser-Trp-Val-Tyr-Ser-Pro-Leu-His-Tyr-Ser-Ala-Arg (peptide designated PN; SEQ ID NO:12) which are encompassed within the C-terminal 25 amino acid residues of PIPKIγ661 and interact with talin at the same site as integrin. A cysteine residue at the N-terminus of the PIPKIγ661 peptides provides a residue which may be modified with a probe, preferably a fluorescent probe. Fluorescent probes may be linked to the cysteine residue using conventional thiol or thiolester linkages. Upon binding to the N-terminal 450 amino acid residues of talin, a PIPKIγ661 peptide of SEQ ID

NO:12 or derivatives thereof exhibited a large change in rotational motion as detected by changes in fluorescent emission anisotropy. It was found that peptides phosphorylated at tyrosines located at Tyr residue 644 (peptide designated pY644), Tyr residue 649 (peptide pY649) or Tyr residue 644 and 649 (peptide designated pY644/649) had a higher binding affinity for talin than did the unphosphorylated peptide (*i.e.*, peptide PN) in a binding assay between talin and integrin (Figure 2). In this assay, an agent which is an inhibitor of the interaction between PIPKIγ661 and talin blocks binding of a peptide of SEQ ID NO:12 and changes the fluorescent emission anisotropy as compared to a control (*e.g.*, binding in the absence of the inhibitor).

When assaying test agents, a control may also include a known agent which has a high affinity for binding and inhibiting the interaction between PIPKIγ661 and talin (*e.g.*, PIPKIγ661 peptides pY644, pY649, and pY644/649) or a known agent which has a low affinity for binding and inhibiting the interaction between PIPKIγ661 and talin (*e.g.*, PIPKIγ661 peptide PN). An agent that modulates the level of bound PIPKIγ661 to talin is one which, for example, blocks binding of a PIPKIγ661 peptide of SEQ ID NO:12 and decreases the fluorescence emission anisotropy of said peptide.

Exemplary fluorescent probes which may be attached to the N-terminus of a PIPKIγ661 peptide are well-known in the art and include, but are not limited to, α-Phycoerythrin, Green Fluorescent Protein, Phycocyanine, Allophycocyanine, Tricolor, AMCA, AMCA-S, AMCA, BODIPY FL, BODIPY 493/503, BODIPY FL Br2, BODIPY R6G, BODIPY 530/550, BODIPY TMR, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY

581/591, BODIPY TR, Cascade Blue, CI-NERF, Dansyl, Dialkylaminocoumarin, 4',6'-Dichloro-2',7'-dimethoxy-fluorescein, 2',7'-dichloro-fluorescein, Cy3, Cy5, Cy7, DM-NERF, Eosin, Eosin F3S, Erythrosin, Fluorescein, Fluorescein Isothiocyanate Hydroxycoumarin, Isosulfan Blue, Lissamine Rhodamine B, Malachite Green, Methoxycoumarin, Napthofluorecein, NBD, Oregon Green 488, Oregon Green 500, Oregon Green 514, Propidium Iodide Phycoerythrin, PyMPO, Pyrene, Rhodamine 6G, Rhodamine Green, Rhodamine Red, Rhodol Green, 2',4',5',7'-Tetrabromosulfonefluorescein, Tetramethyl-rhodamine, Texas Red, X-rhodamine; Lucifer Yellow and the like. Detection of changes in fluorescence may be carried out using such methods as fluorescence spectroscopy, fluorescence resonance energy transfer (FRET), fluorescent lifetime imaging (FLIM) (Lakowicz, *et al.* (1992) *Anal. Biochem.* 202:316-330), or fluorescence polarization.

As PIPKIγ661 and integrin have overlapping binding sites on talin, the ability of a test agent to modulate the binding between talin and integrin also may be assayed to identify the specificity of the agent that modulates the interaction between PIPKIγ661 and talin. In other words, it may be determined whether the agent binds to talin or PIPKIγ661 to modulate their interaction. Accordingly, an integrin which may be used includes a full-length integrin (SEQ ID NO:13) or any fragment which binds to talin (*e.g.*, β1-integrin tail; Cys-Met-Asn-Ala-Lys-Trp-Asp-Thr-Gly-Glu-Asn-Pro-Ile-Tyr-Lys-Ser-Ala; SEQ ID NO:14).

In a screening assay for an agent which modulates the activity of PIPKIγ661 as determined by the phosphorylation of PIPKIγ661 in the presence of Src or Src and FAK, the step of detecting the activity of PIPKIγ661 is carried out by

detecting the presence or absence of phosphorylation of PIPKIγ661. Preferably, the phosphorylation state of Tyr^{644} of PIPKIγ661 is detected. The phosphorylation assay is carried out under suitable assay conditions using well-known methods and a phosphate donor may be added with or after the agent.

It is contemplated that the phosphorylation state of Tyr^{644} of PIPKIγ661 may be determined using a variety of separation and/or detection methods, including those exemplified herein. For example, [^{32}P]phosphorylated PIPKIγ661 is digested with trypsin and separated by well-known conventional column chromatography, 2-D gel electrophoresis, or capillary electrophoresis methodologies. For separation by column chromatography, reverse-phase HPLC may be employed with collection via peak detection. Under the conditions used for reverse-phase HPLC (0.05% TFA, pH 2.2), a phosphorylated peptide generally elutes slightly earlier than the corresponding non-phosphorylated peptide and may or may not be separated from it. Once HPLC fractions containing the phosphorylated Tyr^{644} peptide of PIPKIγ661 are located by Cerenkov counting, a small aliquot of each may be analyzed by MALDI-MS.

As an alternative to radiolabeling, western blots made from 2-D gels may be probed using anti-phosphoserine antibodies (Research Diagnostics, Inc., Flanders, NJ) to recognize the degree of phosphorylation of a peptide fragment of PIPKIγ661 containing Tyr^{644}.

Alternatively, one may use a phosphoprotein purification kit (QIAGEN®, Valencia, CA) for separation of the phosphorylated from the unphosphorylated cellular protein fraction. The affinity chromatography procedure, in which phosphorylated proteins are bound to a column while

unphosphorylated proteins are recovered in the flow-through fraction, reduces complexity and greatly facilitates phosphorylation-profile studies. PIPKIγ661 may then be purified from each fraction and the degree of phosphorylation of PIPKIγ661 determined via autoradiography or immunoassays.

In a preferred embodiment, the phosphorylation of PIPKIγ661 is detected using an antibody which specifically recognizes the phosphorylation state of PIPKIγ661. Preferably, the antibody specifically recognizes the phosphorylation state of tyrosine residue 644 of PIPKIγ661.

An antibody which specifically recognizes the phosphorylation state of Tyr^{644} may be either polyclonal or monoclonal so long as it is able to discriminate between the unphosphorylated and phosphorylated forms of Tyr^{644} and bind PIPKIγ661 to form an PIPKIγ661-antibody complex, *i.e.*, antigen-antibody complex. For example, an antibody which specifically recognizes the unphosphorylated state of Tyr^{644} will only bind to an unphosphorylated Tyr^{644} and not to a phosphorylated Tyr^{644}. Likewise, an antibody which specifically recognizes the phosphorylated state of Tyr^{644} will only bind to a phosphorylated Tyr^{644} and not to an unphosphorylated Tyr^{644}. In a preferred embodiment, the antibody recognizes the phosphorylated form of Tyr^{644} of PIPKIγ661.

Antibodies which may be used to detect the phosphorylation state of Tyr^{644} may be natural or partially or wholly synthetically produced. All fragments or derivatives thereof which maintain the ability to specifically bind to and recognize the phosphorylation state of Tyr^{644} are also contemplated. The antibodies may be a member of any immunoglobulin class, including any of the

classes: IgG, IgM, IgA, IgD, and IgE. Derivatives of the IgG class, however, are preferred in the present invention.

Antibody fragments may be any derivative of an antibody which is less than full-length. Preferably, the antibody fragment retains at least a significant portion of the full-length antibody's specific binding ability. Examples of antibody fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, scFv, Fv, dsFv diabody, or Fd fragments. The antibody fragment may be produced by any means. For instance, the antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody or it may be recombinantly produced from a gene encoding the partial antibody sequence. The antibody fragment may optionally be a single-chain antibody fragment. Alternatively, the fragment may comprise multiple chains which are linked together, for instance, by disulfide linkages. The fragment may also optionally be a multi-molecular complex. A functional antibody fragment will typically comprise at least about 50 amino acids and more typically will comprise at least about 200 amino acids. As used herein, an antibody also includes bispecific and chimeric antibodies.

Naturally produced antibodies may be generated using well-known methods (see, *e.g.*, Kohler and Milstein (1975) Nature 256:495-497; Harlow and Lane, *In:* Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988)). Alternatively, antibodies which specifically recognize the phosphorylation state of Tyr^{644} are derived by a phage display method. Methods of producing phage display antibodies are well-known in the art (*e.g.*, Huse, *et al.* (1989) *Science* 246(4935):1275-81).

Selection of PIPKIγ661-specific antibodies is based on binding affinity to Tyr^{644} which is either phosphorylated or

unphosphorylated and may be determined by the various well-known immunoassays provided herein.

In general, to detect the phosphorylation state of Tyr^{644} using an antibody which specifically recognizes the phosphorylation state of Tyr^{644} involves detecting the formation of an antigen-antibody complex using an immunoassay. Any suitable immunoassay may be used in this method to detect and/or quantitate antigens. Exemplary immunoassays which may be used include, but are not limited to, enzyme-linked immunosorbent, immunodiffusion, chemiluminescent, immunofluorescent, immunohistochemical, radioimmunoassay, agglutination, complement fixation, immunoelectrophoresis, western blots, mass spectrometry, antibody array, and immunoprecipitation assays and the like which may be performed *in vitro*, *in vivo* or *in situ*. Such standard techniques are well-known to those of skill in the art (see, *e.g.*, "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell *et al.*, "Methods and Immunology", W.A. Benjamin, Inc., 1964; and Oellerich, M. (1984) *J. Clin. Chem. Clin. Biochem.* 22:895-904; Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988) 555-612).

These immunoassays typically rely on labeled antigens, antibodies, or secondary reagents for detection. These proteins may be labeled with radioactive compounds, enzymes, biotin, or fluorochromes. Of these, radioactive labeling may be used for almost all types of assays. Enzyme-conjugated labels are particularly useful when radioactivity must be avoided or when quick results are needed. Biotin-coupled reagents usually are detected with labeled streptavidin. Streptavidin binds tightly and quickly to biotin and may be labeled with radioisotopes or

enzymes. Fluorochromes, although requiring expensive equipment for their use, provide a very sensitive method of detection. Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to antibodies or fragments thereof may be accomplished using standard techniques (see, for example, Kennedy, *et al.* (1976) *Clin. Chim. Acta* 70:1-31 and Schurs, *et al.* (1977) *Clin. Chim Acta* 81:1-40).

In accordance with detecting the phosphorylation state of Tyr^{644}, the presence or absence of the antigen-antibody complex is correlated with phosphorylation of Tyr^{644}. For example, an agent which blocks the binding of an antibody which specifically recognizes phosphorylated Tyr^{644}, is indicative of an agent which blocks the phosphorylation of PIPKIγ661 by Src. Conversely, an agent which increases or stimulates, for example, the rate of phosphorylation of PIPKIγ661 by Src will increase or enhance the rate of binding of an antibody which specifically recognizes phosphorylated Tyr^{644}.

Another aspect of the present invention is a method for identifying an agent that modulates cell focal adhesion assembly. This cell-based assay involves contacting a cell which lacks active PIPKIγ661 or which overexpresses PIPKIγ661 with a test agent and measuring the adherence of the cell to a surface as compared to a same cell type which has not been contacted with the test agent. Agents which inhibit or decrease the adherence of a cell which overexpresses PIPKIγ661 are useful for treating conditions or diseases such as cancer invasiveness or cancer metastasis. Agents which increase, enhance, or stimulate adherence of a cell which lacks active PIPKIγ661 are useful

for treating conditions or diseases involving wound healing, immune responses, or neuronal development. A cell which lacks active PIPKIγ661 may include a cell which expresses a kinase dead PIPKIγ661 or lacks expression of PIPKIγ661 either by using a gene knock out or inhibitory RNA approach. Promoters, vectors, and cell lines for expressing a PIPKIγ661, a kinase dead PIPKIγ661, or inhibitory RNA are provided herein. Methods of generating a cell line with a gene knock-out are well-known in the art.

Adherence of a cell to a surface (*e.g.*, a membrane or petri plate) may be determined by washing experiments or by microscopic analysis. Washing experiments, for example, may be conducted by passing a medium over the cells on the surface and measuring the number of cells adhering in the presence and absence of a test agent. An increase in the number of cells which adhere in the presence of the agent is indicative of said agent enhancing or increasing cell adhesion. Conversely, an increase in the number of cells which do not adhere in the presence of the agent is indicative of said agent inhibiting or decreasing cell adhesion.

Alternatively, antibodies directed to focal adhesion proteins (*e.g.*, PIPKIγ661 or talin) may be used to label focal adhesions in a determination of focal adhesion size. Overexpression of PIPKIγ661 increases the size of focal adhesions and, concurrently, the adherence of these cells. Hence, an agent which decreases the size of focal adhesions in a cell overexpressing PIPKIγ661, would accordingly decrease the adherence of the cell as well. Methods for observing focal adhesions in a cell are exemplified herein.

Agents which modulate the activity of PIPKIγ661 or cell focal adhesion assembly may be identified by screening a

library of test agents. Agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A library may comprise either collections of pure agents or collections of agent mixtures. Examples of pure agents include, but are not limited to, peptides, polypeptides, antibodies, oligonucleotides, carbohydrates, fatty acids, steroids, purines, pyrimidines, lipids, synthetic or semi-synthetic chemicals, and purified natural products, derivatives, structural analogs or combinations thereof. Examples of agent mixtures include, but are not limited to, extracts of prokaryotic or eukaryotic cells and tissues, as well as fermentation broths and cell or tissue culture supernates. In the case of agent mixtures, one may not only identify those crude mixtures that possess the desired activity, but also monitor purification of the active component from the mixture for characterization and development as a therapeutic drug. In particular, the mixture so identified may be sequentially fractionated by methods commonly known to those skilled in the art which may include, but are not limited to, precipitation, centrifugation, filtration, ultrafiltration, selective digestion, extraction, chromatography, electrophoresis or complex formation. Each resulting subfraction may be assayed for the desired activity using the original assay until a pure, biologically active agent is obtained.

Agents of interest in the present invention are those with functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic

structures substituted with one or more of the above functional groups.

Alternatively, peptide derivatives of the sequence of PIPKIγ661, PIPKIγ661 peptides PN, pY644, pY649, pY644/649, or talin may be designed to modulate the interaction between PIPKIγ661 and talin.

Agents which modulate the activity of PIPKIγ661 or cell focal adhesion assembly are useful as therapeutic agents for preventing or treating a cell migration-mediated condition or disease. Accordingly, another aspect of the present invention is a method for preventing or treating a cell migration-mediated condition or disease in a subject by administering to a subject an effective amount of an agent that modulates the activity of PIPKIγ661 or cell focal adhesion assembly in a cell lacking active PIPKIγ661 or overexpressing PIPKIγ661. Cell migration-mediated conditions or diseases which may be prevented or treated in accordance with the method of the invention include, but are not limited to, cancer invasiveness; cancer metastasis; wound healing; neuronal development (*e.g.*, migration of axons in spinal cord injury); neuronal disorders (*e.g.*, Hirschsprung's Disease); immune responses (*e.g.*, macrophage migration); and immune disorders (*e.g.*, Wiskott-Aldrich Syndrome). Whether the agent stimulates or enhances or inhibits or decreases the activity of PIPKIγ661 or cell focal adhesion assembly to provide a therapeutic effect will be dependent on the cell migration-mediated condition or disease. Preferably, an agent which stimulates or enhances the activity of PIPKIγ661 or cell focal adhesion assembly will be used to prevent or treat a condition or disease which has reduced cell migration or may benefit from a stimulation or enhancement of cell migration (*e.g.*,

in wound healing, neuronal development and immune responses). Further, it is preferable that an agent which inhibits or decreases the activity of PIPKIγ661 or cell focal adhesion assembly will be used to prevent or treat a condition or disease which has increased cell migration or may benefit from a decrease or inhibition of cell migration (*e.g.*, cancer invasiveness or cancer metastasis).

A subject having, or at risk of having, a cell migration-mediated condition or disease may be treated in accordance with the method of the present invention. A subject at risk of having a cell migration-mediated condition or disease includes individuals who have a high probability of developing the condition or disease (*e.g.*, metastasis of an existing cancer) or which may have inherited the condition or disease and may benefit from a preventive therapy.

An effective amount of an agent which modulates the activity of PIPKIγ661 or cell focal adhesion assembly is an amount which prevents, eliminates or alleviates at least one sign or symptom of a cell migration-mediated condition or disease. Signs or symptoms associated with a cell-migration-mediated condition or disease vary with the condition or disease being prevented or treated and are well-known to the skilled clinician. Examples of signs and/or symptoms of cancer metastasis that may be monitored to determine the effectiveness of an agent which modulates the activity of PIPKIγ661 or cell focal adhesion assembly include, but are not limited to, tumor size, feelings of weakness, and pain perception. The amount of the agent required to achieve the desired outcome of preventing, eliminating or alleviating a sign or symptom of a cell migration-mediated condition or disease will be dependent

on the pharmaceutical composition of the agent, the patient and the condition of the patient, the mode of administration, and the type of condition or disease being prevented or treated.

A pharmaceutical composition is one which contains the agent and a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier is a material useful for the purpose of administering the medicament, which is preferably sterile and non-toxic, and may be solid, liquid, or gaseous materials, which is otherwise inert and medically acceptable, and is compatible with the active ingredients. A generally recognized compendium of methods and ingredients of pharmaceutical compositions is Remington: The Science and Practice of Pharmacy, Alfonso R. Gennaro, editor, 20th ed. Lippincott Williams & Wilkins: Philadelphia, PA, 2000.

A pharmaceutical composition may contain other active ingredients such as preservatives. A pharmaceutical composition may take the form of a solution, emulsion, suspension, ointment, cream, granule, powder, drops, spray, tablet, capsule, sachet, lozenge, ampoule, pessary, or suppository. It may be administered by continuous or intermittent infusion, parenterally, intramuscularly, subcutaneously, intravenously, intra-arterially, intrathecally, intraarticularly, transdermally, orally, bucally, intranasally, as a suppository or pessary, topically, as an aerosol, spray, or drops, depending upon whether the preparation is used to treat an internal or external condition or disease. Such administration may be accompanied by pharmacologic studies to determine the optimal dose and schedule and would be within the skill of the ordinary practitioner.

An agent which modulates the activity of PIPKIγ661 or cell focal adhesion assembly and is useful as a therapeutic agent for preventing or treating a cell migration-mediated condition or disease may be identified using the screening assays provided herein or may also include agents which modulate the expression of PIPKIγ661 or talin. For example, the expression of PIPKIγ661 may be inhibited using inhibitory RNAs such as ribozymes, antisense RNA, RNAi, siRNA and the like. These RNA molecules may be designed to specifically interact with the nucleic acid sequences encoding PIPKIγ661 to decrease the expression of PIPKIγ661 thereby decreasing its capacity to bind to talin. As the RNA molecule encoding PIPKIγ661 is alternatively spliced and is unique from other PIPKIγ RNA molecules by containing exon 17, it is preferable that the inhibitory RNA molecule is directed to exon 17 of the gene encoding PIPKIγ661. Specific inhibitory RNA molecules may be selected experimentally or empirically. For example, siRNA target sites in exon 17 may be 19-27 nucleotides in length, include an AA dinucleotide sequence at the 5′ end and preferably have a G/C content of 30-50% (see, *e.g.*, Elbashir, *et al.* (2001) *Nature* 411: 494-498).

It is further contemplated that an agent which modulates the activity of PIPKIγ661 or cell focal adhesion assembly may be attached to a targeting moiety which delivers the agent to a cell-type or tissue of interest to decrease potentially harmful side-effects of modulating the activity of PIPKIγ661 or cell focal adhesion assembly in all cell-types or tissues. For example, a targeting moiety to a cancerous tumor may include peptide hormones such as bombesin, stomatostatin and luteinizing hormone-releasing hormone (LHRH) or analogs thereof. Cell-surface receptors

for peptide hormones have been shown to be overexpressed in tumor cells (Schally (1994) *Anti-Cancer Drugs* 5:115-130; Lamharzi, *et al.* (1998) *Int. J. Oncol.* 12:671-675) and the ligands to these receptors are known tumor cell targeting agents (Grundker, *et al.* (2002) *Am. J. Obstet. Gynecol.* 187(3):528-37; WO 97/19954).

It is further contemplated that exogenous application or moderate overexpression of PIPKIγ661 may also have therapeutic value in treating or preventing a cell migration-mediated condition or disease.

The invention is described in greater detail by the following non-limiting examples.

Example 1: Generation of Constructs.

Site-directed mutagenesis for creation of kinase-dead PIPKIγ661 (D253A) was performed using PCR-primer overlap extension with mutagenic primers (Kunz, *et al.* (2000) *supra*). The mutagenic primers were 5'-CAA AAT GCA CCT TAA GTT CGC CCT CAA GGG CTC CAC-3' (SEQ ID NO:15) and 5'-GTA CGT GGA GCC CTT GAG GGC GAA CTT AAC CTG C-3' (SEQ ID NO:16). The flanking primers were 5'-CTA TGC ACC TGT TGC CTT CCG CTA CTT C-3' (SEQ ID NO:17) and 5'-GGC CGT GGA ATA CAG AGC CTT C-3' (SEQ ID NO:18). The mutation was confirmed by DNA sequence analysis. A 0.5-kilobase *Xma*I-*Cla*I restriction fragment was used to replace the corresponding restriction fragment in the HA-PIPKIγ661/pcDNA3 construct.

To construct PIPKIα/γ661 and PIPKIα/γ635 chimeras, a *Pml*I blunt cloning site was created after amino acid 444 of PIPKIα in pET28b (Novagen, Madison, WI) using well-established methods (Loijens and Anderson (1996) *J. Biol. Chem.* 271:32937-32943) with primers 5'-GCG TGA ACG GTT CAA GCG CTT CAC GTG CAA CAC AG-3' (SEQ ID NO:19) and 5'-CTT AAA

TAC TGT GTT GAC CAT GAA GCG CTG G-3′ (SEQ ID NO:20). The resulting construct was subsequently digested with *Pml*I and *Eco*RI. PIPKIγ661 and 635 C-terminal fragments (178 and 153 amino acids, respectively) were amplified by PCR from the corresponding HA-tagged pcDNA3 constructs, digested with *Eco*RI and ligated into the restricted PIPKIα pET28b vector. To construct the PIPKIα/γ26 chimera, a *Pml*I site was created immediately before the stop codon of PIPKIα in the pET28b using PCR primers 5′-CCC TTA AGC AGT GAA ACA CAG TAC TCA GTT G-3′ (SEQ ID NO:21) and 5′-CCC CCA CGT GGG TGA ACT CTG ACT CTG-3′ (SEQ ID NO:22). The resulting construct was then digested with *Pml*I and *Eco*RI. The PIPKIγ26 fragment was PCR amplified from the HA-tagged PIPKIγ661/pcDNA3 construct, digested and ligated into the PIPKIα vector described above. All three chimeras were then subcloned from pET28b into the pCMV TAG 3 (Stratagene, La Jolla, CA) mammalian expression vector and sequenced. GFPIγ26 was constructed by PCR amplification of the C-terminal 26 amino acids and 3′UTR of PIPKIγ661 with primer 5′-GCT CAA GCT TCG AAT TCT CCC ACC GAC GAG AGG-3′ (SEQ ID NO:23) and the vector primer SP6. An *Eco*RI site (underlined) was incorporated by PCR. The PCR product and EGFP vector was digested with *Eco*RI and *Apa*I. The GFPIγ26 construct was confirmed by sequencing.

Cloning of PIPKIγ661 into a bacterial expression vector was performed by liberating PIPKIγ661 from the HA-tagged PIPKIγ661 vector using *Sal*I/*Not*I and subcloning into pET28c. The C-terminal 178 amino acid of PIPKIγ661 was obtained by PCR, digested with *Bam*HI and *Eco*RI and subcloned into pET28c.

Human talin1 head region (1-435) was cloned from HEK293T cells by PCR and subcloned into pET42. The C-

terminal truncated or site-directed mutagenesis for the PIPKIγ661 mutants or talin head was performed using PCR-primer overlap extension with mutagenic primers. PIPKIγ661 mutants were then subcloned into pcDNA3.1 and talin head mutants were subcloned into pET42. His-tagged mouse β1-integrin tail was made using well-established methods (Pfaff, *et al.* (1998) *supra*) and subcloned into pET28. The C-terminus of PIPKIγ635 (439-635), PIPKIγ661 (439-661), or the Tyr to Phe mutants of PIPKIγ661 were PCR amplified from the corresponding pcDNA3.1 constructs and subcloned into pET28. All mutants were confirmed by DNA sequence analysis. FAK, FAKTyr397Phe, kinase dead FAK and c-Src constructs were generated using well-known methods.

Example 2: Yeast Two-Hybrid Screen

The yeast two-hybrid screen was performed according to well-known methods (James, *et al.* (1996) *Genetics* 144:1425-1436). cDNA libraries which were screened include mouse embryonic, human B cell, human breast, human prostate, human placenta and mouse brain.

Example 3: Kinase Assays

Activity of purified recombinant or immunoprecipitated PIPKI proteins was assayed against 25 μM phosphotidylinositol-4-phosphate (PI4P) micelles using well-known methods (Kunz, *et al.* (2000) *supra*).

For *in vitro* Src kinase assays, primary chicken embryo (CE) cells were prepared and maintained as described (Reynolds (1987) *EMBO J.* 6:2359-2364). Exogenous c-SrcTyr527Phe was expressed using the RCAS B replication competent avian retroviral vector as described (Schaller, *et al.* (1999) *supra;* Reynolds (1987) *supra*). Cells were

transfected with LIPOFECTAMINE™ (Life Technologies, Inc., Rockville, MD) and LIPOFECTAMINE™ Plus (Life Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. Approximately 7-9 days post-transfection of RCAS B/c-Src, cells were lysed.

c-Src was immunoprecipitated from ~1 mg CE cell lysates using the EC10 monoclonal antibody and Protein A-SEPHAROSE (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hour at 4°C. Immune complexes were washed twice with lysis buffer, twice with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) and twice with kinase reaction buffer (20 mM PIPES, pH 7.2; 5 mM $MnCl_2$; 5 mM $MgCl_2$). The immune complexes were resuspended in kinase reaction buffer containing 35 µM ATP including 10 µCi of [γ-^{32}P] ATP (6000 Ci $mmol^{-1}$; Perkin Elmer LifeSciences, Boston, MA), and incubated with 4 µg of recombinant substrates for various times at room temperature. Reactions were terminated by adding sample buffer. Samples were resolved by SDS-PAGE and the gels then stained with SYPRO® Orange (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The stained gels were analyzed for fluorescence and by phosphorimaging after drying, using a MOLECULAR DYNAMICS STORM® PHOSPHORIMAGER® (Sunnyvale, CA).

Example 4: Cell Culture, Transfection, Immunofluorescence and Confocal Microscopy

HEK 293T cells and NRK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (INVITROGEN™, Carlsbad, CA) and antibiotics. NRK cells were transfected using FUGENE™ 6 (Roche, Indianapolis, IN) according to manufacturer's instructions for 24 hours. HEK293T cells in 1 µg/ml collagen-coated dishes were

transfected using 2 µg PIP kinase expression vector together with 3-4 µg empty pcDNA3 vector, 3 µg CD2FAK or FRNK, 2 µg empty pcDNA3 vector plus 2 µg wild-type or mutant FAK, 2 µg empty pcDNA3 vector plus 2 µg c-Src, or 2 µg wild-type or mutant FAK plus 2 µg c-Src as indicated, using the well-known calcium phosphate-DNA coprecipitation method for 48 hours.

Immunofluorescence and confocal microscopy were performed as is well-known in the art (Kunz, *et al.* (2000) *supra*). Z series were created by sequentially scanning green and red channels at 0.2 µm steps.

Example 5: Expression and Purification of PIP Kinase in *Escherichia coli*

pET28 constructs containing His-tagged PIPKIα, PIPKIγ661, wild-type or mutant C-terminal tails of PIPKIγ661, β1-integrin tail, or pET42 constructs containing wild-type or mutant talin head regions were transformed into BL21(DE3) (Novagen, Inc., Madison, WI). Proteins were expressed and purified using His-resin following the manufacturer's instructions (Novagen, Inc., Madison, WI).

Example 6: Antibodies

Anti-talin, anti-vinculin and anti-Flag (M2) antibodies were obtained from Sigma (St. Louis, MO). Monoclonal mouse anti-PY (4G10), anti-FAK (4.47), anti-Src (EC10) and anti-paxillin (5H11) were from Upstate Group, Inc. (Charlottesville, VA). Polyclonal rabbit anti-PY antibody was obtained from Transduction Laboratories (Lexington, KY). Anti-HA and anti-c-Myc antibodies were from Covance (Harrogate UK). HRP-conjugated anti-GST antibody was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Anti-His and HRP-conjugated anti-T7

antibodies were from INVITROGEN™ (Carlsbad, CA). Polyclonal PIPKIγ anti-serum was generated using purified His-tagged PIPKIγ661. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Anti-serum was purified on an affinity column generated by coupling recombinant C-terminus of PIPKIγ661 to cyanogen bromide-activated Sepharose 4B (Sigma, St. Louis, MO)(Loijens and Anderson (1996) *supra*).

Example 7: Immunoprecipitation and Immunoblotting

Immunoprecipitation was performed using standard methods (Zhang, *et al.* (1997) *J. Biol. Chem.* 272:17756-17761). Briefly, transfected HEK293T cells were harvested and lysed in Buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1-1.0% NP-40, 5.0 mM NaF, 2 mM Na_3VO_4, 4 mM $Na_2P_2O_7$, 1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10% glycerol), centrifuged and incubated with protein A-Sepharose and 5 µg anti-HA, anti-c-Myc, anti-Flag, anti-PIPKIγ or anti-talin antibody as indicated at 4°C for 4 hours to overnight. The immunocomplexes were washed with Buffer A, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed as indicated. For the kinase assay, the immunocomplexes were washed and stored in kinase buffer.

Example 8: Cell Stimulation and Spreading

Transfected HEK 293T cells were washed with phosphate-buffer saline (PBS) and detached with 0.25% trypsin at 37°C. The cells were harvested and washed with PBS, and suspended in serum-free DMEM at 37°C for 1 hour. After stimulation with 10 ng/ml platelet-derived growth factor (PDGF) (R&D Systems Inc., Minneapolis, MN), 1 µM LPA

(Sigma, St. Louis) or serum at 37°C for 15 minutes, cells were lysed as for immunoprecipitation. For spreading assays, transfected NRK cells were detached with 1 mM EDTA, washed with PBS, and subsequently $0.1x10^6$ cells were plated on 1 µg/ml collagen-coated coverslips in serum-free DMEM. After the indicated time, the coverslips were fixed and stained as described herein.

Example 9: Peptide Synthesis and GST Pull-Down Assays

Phosphorylated or non-phosphorylated peptides relevant to PIPKIγ661, named as PN (Cys-Asp-Glu-Arg-Ser-Trp-Val-Tyr-Ser-Pro-leu-His-Tyr-Ser-Ala-Arg; SEQ ID NO:12), pY644 (Cys-Asp-Glu-Arg-Ser-Trp-Val-*Tyr-Ser-Pro-Leu-His-Tyr-Ser-Ala-Arg; SEQ ID NO:12), pY649 (Cys-Asp-Glu-Arg-Ser-Trp-Val-Tyr-Ser-Pro-Leu-His-*Tyr-Ser-Ala-Arg; SEQ ID NO:12), and pY644/649 (Cys-Asp-Glu-Arg-Ser-Trp-Val-*Tyr-Ser-Pro-Leu-His-*Tyr-Ser-Ala-Arg; SEQ ID NO:12)), wherein "*" indicates phosphorylation, were synthesized as >95% purity (INVITROGEN™, Carlsbad, CA). The peptides corresponding to β1-integrin tail, named as β1InPN (Cys-Met-Asn-Ala-Lys-Trp-Asp-Thr-Gly-Glu-Asn-Pro-Ile-Tyr-Lys-Ser-Ala; SEQ ID NO:14), β1InpT (Cys-Met-Asn-Ala-Lys-Trp-Asp-*Thr-Gly-Glu-Asn-Pro-Ile-Tyr-Lys-Ser-Ala; SEQ ID NO:14), β1InpY (Cys-Met-Asn-Ala-Lys-Trp-Asp-*Thr-Gly-Glu-Asn-Pro-Ile-*Tyr-Lys-Ser-Ala; SEQ ID NO:14), and β1InpTpY (Cys-Met-Asn-Ala-Lys-Trp-Asp-*Thr-Gly-Glu-Asn-Pro-Ile-*Tyr-Lys-Ser-Ala; SEQ ID NO:14), were synthesized as >97% purity (See http://biotech.wisc.edu/ServicesResearch/Peptide/PeptideSynth/).

Purified GST-talin head proteins were incubated with PIPKIγ661 tails or β1-integrin tail, together with Glutathione SEPHAROSE™ 4 Fast Flow (Amersham Biosciences,

Piscataway, NJ), in Buffer B (PBS, 0.2% NP-40, 2 mM DTT) at 4°C for 4 hours. The beads were washed three times with Buffer B and analyzed by western blot. For competition assays, GST-talin head proteins were incubated with peptides or competing proteins for 2 hours, then the binding proteins were added and the mixtures were incubated for another 4 hours.